

Identification of Blood Meals Imbided by Phlebotomine Sand Flies Using Cytochrome *b* PCR and Reverse Line Blotting

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Abstract

Blood meal identification is important for determining the host preferences and the vectorial capacity of hematophagous arthropods. In the past, mostly serological techniques using host-specific antibodies were used, but in recent years more sensitive and accurate polymerase chain reaction (PCR)-based molecular approaches for identifying blood meals have been developed. Here, a vertebrate-specific PCR is combined with reverse line blot analysis for identifying blood meals ingested by female phlebotomine sand fly vectors of leishmaniasis. Species-specific oligonucleotides were covalently linked to nylon membranes, and biotinylated PCR products of the mitochondrial cytochrome *b* gene were used as probes in a hybridization reaction revealed using colorimetric or enhanced chemiluminescent detection systems. This combination identified blood meals up to 96 hours after ingestion containing minimal amounts of DNA (>0.1 pg). The specific probes discriminated between putative host species in several study areas. The source of blood was identified in 68 of 89 wild-caught sand flies tested (76%). Mixed blood meals were identified in 15 (17%) of those. The advantages and limitations of this method are discussed.

Key Words: Leishmania; Sand fly (flies); Zoonosis

Introduction

IDENTIFICATION OF BLOOD MEALS ingested by hematophagous arthropods is important for determining their host preferences and hence, their vectorial capacity for disease agents (Boreham 1975, Boakye et al. 1999). Because most *Leishmania* spp. are zoonotic pathogens, characterizing the feeding habits of endemic phlebotomine sand flies is crucial for incriminating putative vectors, elucidating natural transmission cycles, and developing efficacious control strategies (Killick-Kendrick 1990, 1999, Alexander and Maroli 2003).

Different serological methods have been used to identify blood sources of hematophagous arthropods (Washino and Tempelis 1983). Initially, precipitin test and hemagglutination inhibition assays prevailed (Ogusuku et al. 1994, Boreham 1975). Later, the enzyme linked immunosorbent assay (ELISA) largely replaced the older tests (Beier et al. 1988, Gomez et al. 1998, Gomes et al. 2001, Svobodova et al. 2003). Inherent limitations of these methods include the need to produce species-specific antibodies against each potential animal host and the requirement for relatively fresh blood (Blackwell et al. 1994). The introduction of a polymerase chain reaction (PCR)-based molecular methods has simpli-

fied and improved the sensitivity of blood meal identification (Mukabana et al. 2002). Several methods involve the amplification of either mitochondrial or nuclear DNA by PCR followed by species identification using restriction digestion (PCR-RFLP), terminal restriction length polymorphisms, heteroduplex mobility assays, and sequencing (Boakye et al. 1999, Ngo and Kramer 2003, Meece et al. 2005, Steuber et al. 2005, Haouas et al. 2007). All of these approaches require relatively large amounts of PCR product and do not detect multiple blood sources within a single insect. Recently, a reverse line blot (RLB) assay was successfully used for identifying the sources of blood ingested by *Ixodes ricinus* ticks (Humair et al. 2007).

Our study describes a blood meal identification approach based on PCR amplification of the mitochondrial cytochrome *b* gene (*cyt b*) followed by RLB analysis. RLB is a highly reproducible technique in which species-specific oligonucleotides are covalently linked to nylon membranes through the formation of amide bonds between the carboxyl group present on the membrane and amino-linked oligonucleotides. Biotinylated PCR products of the mitochondrial *cyt b* gene were used as probes in a hybridization reaction revealed using colorimetric or enhanced chemiluminescent

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TABLE 1. SPECIES-SPECIFIC OLIGONUCLEOTIDE PROBES DESIGNED TO IDENTIFY DIFFERENT VERTEBRATE BLOOD TYPES BASED ON THEIR HYBRIDIZATION WITH THE CYT B PCR PRODUCT

	Species	Oligonucleotide sequence	Temperature (°C)
1	Human (<i>Homo sapiens</i>)	ATG CAC TAC TCA CCA GAC GC	59.7
2	Donkey (<i>Equus asinus</i>)	CTA CTT TTC ACA GTT TAG CTA CA	55.3
3	Goat (<i>Capra hircus</i>)	ATA CAT ATC GGA CGA GGT CTA	56.0
4	Sheep (<i>Ovis aries</i>)	TCC TAT TTG CGA CAA TAG CTT CCT	58.7
5	Cow (<i>Bos taurus</i>)	ATT ATG GGT CTT ACA CTT T	48.7
6	Gazelle (<i>Gazella gazella</i>)	GAA ACA TGG AAT ATC GGA G	53.1
7	Camel (<i>Camelus dromedaries</i>)	CGT TGG AAT TGT TTT ATT	48.9
8	Canid species	CAG ATT CTA ACA GGT TTA	47.9
9	Domestic cat (<i>Felis domesticus</i>)	CAT TGG AAT CAT ACT ATT	45.6
10	Rock hyrax (<i>Procapra capensis</i>)	CCT ATT CTT CGT ATG TCT TTA	52.1
11	Porcupine (<i>Hystrix indica</i>)	ACA CTG CCT ACA CAA CTA CA	55.6
12	Brown rat (<i>Rattus rattus</i>)	CAG TCA CCC ACA TCT GC	43.1
13	Domestic mouse (<i>Mus musculus</i>)	TGG AGT ACT TCT ACT GTT CGC AGT	60.4
14	Domestic chicken (<i>Gallus gallus</i>)	CAT CCG GAA TCT CCA C	53.7
15	House sparrow (<i>Passer domesticus</i>)	GAG ACG TCC AAT TCG GAT	54.8
16	Rock partridge (<i>Alectoris graeca</i>)	TCA CCG GCC TCC TCC TA	59.1
17	Rock pigeon (<i>Columbia livia</i>)	ACT ACT CGC CGC ACA TTA	54.8
18	Turkey (<i>Meleagris gallopavo</i>)	CTT CTG TGG CCA ACA CAT	54.8
19	General avian probe 1	TAC ACA GCA GAC AC	46.4
20	General avian probe 2	GCC TCA TTC TTC TTC AT	49.4

(ECL) detection systems (Saiki et al. 1989, Zhang et al. 1991). PCR-RLB enabled the detection of single-base differences among homologous DNA segments or genes and has been widely used in characterizing single nucleotide mutations underlying thalassemias (Sutcharitchan et al. 1995, Winichagoon et al. 1999). Because it is so sensitive, templates for RLB must be highly conserved genes displaying very low within-species sequence variation such as the mitochondrial *cyt b* gene. We describe the development of a PCR-RLB blood source detection system for phlebotomine sand flies and discuss the strengths and limitations of this new approach.

Materials and Methods

Collection of sand flies

Sand flies were collected from different locations using CDC light traps, and kept at -20°C until DNA extraction. DNA was extracted from the abdomens of blood-fed flies. Heads and terminalia were mounted in Hoyer's medium and identified to species level using taxonomic keys (Artemiev 1980, Lewis 1982).

Experimental blood feeding for time course analysis

Four-day-old laboratory-reared *Phlebotomus papatasi* were fed for 30 minutes on the skin of a human volunteer or an anesthetized chicken. Sand flies were kept at $26 \pm 1^{\circ}\text{C}$ and 80% relative humidity and maintained on 30% sucrose solution. Flies were collected at different times after feeding (0–96 hours), anesthetized using CO_2 , and frozen in individual microcentrifuge tubes.

DNA extraction

DNA was extracted from vertebrate blood or tissue samples as well as insects by digestion in a total volume of 200

μL of lysis buffer (50 mM NaCl, 10 mM ethylenediaminetetraacetic acid [EDTA], 50 mM Tris-HCl pH 7.4, 1% triton X-100, and 200 $\mu\text{g}/\text{mL}$ of proteinase K). This was followed by extraction with phenol-chloroform and precipitation using ethanol. The precipitated DNA was suspended in Tis-EDTA (TE, 10 mM Tris-HCl pH 7.4, 1 mM EDTA) buffer at a concentration of 10 $\text{ng}/\mu\text{L}$ for vertebrate tissues and in a total volume of 50 μL for sand flies.

Species-specific probes

Species-specific 5'-amino-linked oligonucleotide probes were designed after alignments of mammalian and avian vertebrate *cyt b* sequences obtained from GenBank or by direct sequencing of *cyt b* PCR amplified products (Table 1). General avian probes were designed to include a nucleotide sequence common to all avian species. The selected oligonucleotides were covalently linked to a nylon membrane (see below).

Polymerase chain reaction

Universal primers were designed for the amplification of 344 base pairs of the conserved region of the *cyt b* gene. The direct primer was named Cyto1: 5'-CCA TCA AAC ATC TCA GCA TGA TGA AA-3' at position 14830 in the mitochondrial DNA and the reverse primer was named Cyto2: 5'-CCC CTC AGA ATG ATA TTT GTC CTC-3' at position 15174. Both primers were modified by the addition of a biotin group at their 5'-end. The 344-bp *cyt b* segment was amplified using 1 U of Taq DNA polymerase (Sigma, St. Louis, MO) in a total reaction volume of 50 μL consisting of 75 mM KCl, 10 mM Tris-HCl pH 8.8, 1.5 mM MgCl_2 , 200 μM deoxyribonucleotide triphosphates, 25 pmoles of each primer, and genomic DNA. The thermo-cycling conditions consisted of 35 cycles at 94°C for 30 sec, 55°C for 30 sec, and an elon-

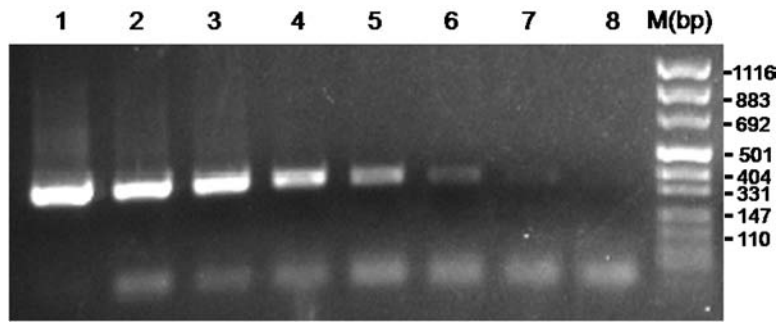


FIG. 1. Sensitivity test of the cytochrome *b* polymerase chain reaction assay at different concentrations of human genomic DNA. Lane 1, 10 ng; lane 2, 1 ng; lane 3, 0.1 ng; lane 4, 10 pg; lane 5, 1 pg; lane 6, 0.1 pg; lane 7, 0.01 pg; and lane 8, no DNA. M, size marker (bp).

gation step at 72°C for 1 min. The PCR amplified products were used as probes in RLB hybridization reactions followed by colorimetric or ECL detection as indicated below.

Immobilization of oligonucleotides to the membranes

The synthetic 5'-end amino modified oligonucleotide probes were covalently linked to nylon membranes through the formation of amide bonds between the carboxyl groups on the nylon and the amino groups linked to the oligonucleotides. Biodyn C (Pall Biomedical, Fajardo, Puerto Rico) nylon membrane were activated in 0.1 N HCl for 5 min, rinsed with DH₂O and soaked in 10% 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) (Sigma, St. Louis, MO, USA) for 15 minutes. The membranes were rinsed in DH₂O water and air dried. Species-specific, 5'-end amino modified oligonucleotides (Table 1) were diluted to 5 pmoles/ μ L and applied to the membrane in line format using a manifold blotter apparatus (Immunetics, Cambridge, MA).

Hybridization and detection

The membrane sheets with the oligonucleotide probes were cut into strips at a right angle to the direction of the blots so that every strip contained a section with each probe. Strips were incubated in prehybridization solution (2 \times SSC [0.15 M NaCl, 0.015 M sodium citrate], 0.1% sodium dodecyl sulphate [SDS]) for 30 min at 45°C with gentle shaking. Biotinylated PCR products were denatured by boiling for 5 min and applied to the membrane strips. Hybridization was performed at 43°C for 1 h followed by a single wash with 0.7 \times SSC, 0.1% SDS for 20 minutes. Hybridized biotinylated DNA was detected by incubating the strips in streptavidin-horseradish peroxidase (HRP; diluted in 2 \times SSC, 0.1% SDS) for 30 min at room temperature. Strips were washed briefly 3 times in 2 \times SSC, 0.1% SDS. For chromogenic detection, a freshly prepared solution containing 0.1 mg/mL of 3,3',5,5' tetramethylbezidine (Sigma), 0.003% H₂O₂ in 0.1 M sodium citrate (pH 5.0) was added. ECL detection was performed

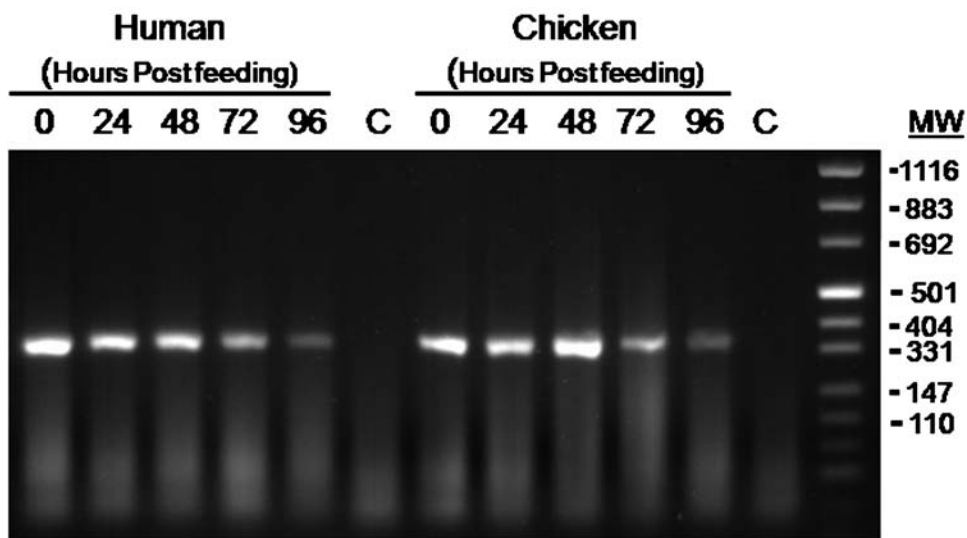


FIG. 2. Cytochrome *b* polymerase chain reaction targeting DNA extracted from sand flies at different times after blood feeding on a human or a chicken. Lane 1, immediately after feeding; lane 2, after 24 hours; lane 3, after 48 hours; lane 4, after 72 hours; lane 5, after 96 hours; and lane 6, no DNA. M: size marker (bp).

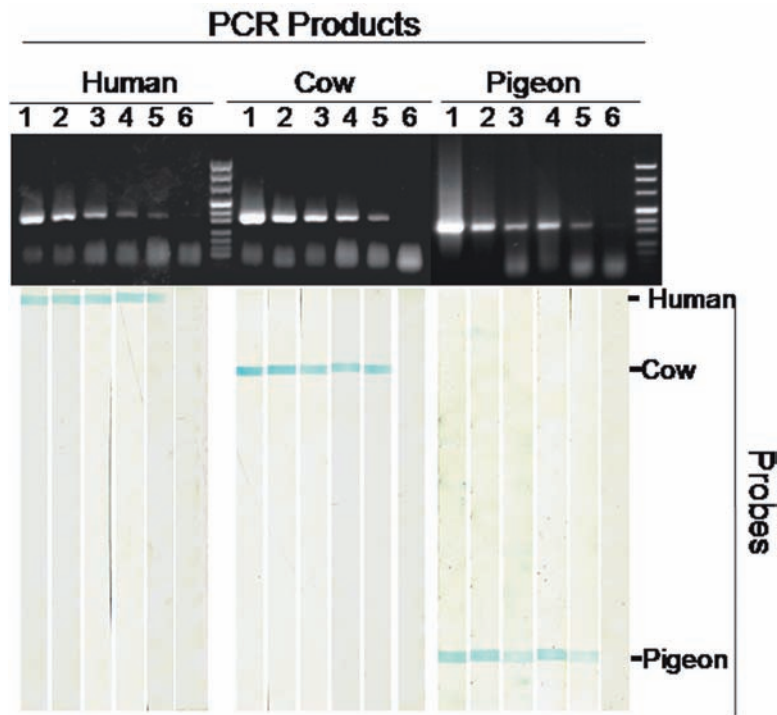


FIG. 3. Sensitivity tests of the cytochrome *b* polymerase chain reaction assay (**top panel**) and corresponding reverse line blot (RLB; **bottom panel**). DNA of three vertebrate species was tested at different concentrations. Lane 1, 10 ng; lane 2, 1 ng; lane 3, 0.1 ng; lane 4, 10 pg; lane 5, 1 pg; and lane 6, no DNA. Sensitivity of the RLB clearly corresponded with that seen on the gel.

immediately after streptavidin-HRP incubation and washing steps using EZ-ECL detection kit (Biological Industries, Beit Haemek, Israel).

Results

PCR assay sensitivity and time-course analysis of blood fed sand flies

In our hands, the *cyt b* PCR primers previously described by Kocher et al. (1989) were not sensitive enough to amplify vertebrate DNA extracted from blood-fed sand flies (data not shown). Therefore, new primers were designed that detected minute quantities of DNA (>0.1 pg; Fig. 1). This is important because sand flies take very small blood meals (around 0.1 μ L) that are digested rapidly, and most cells in mammalian blood do not contain nuclei. The amplification limit of the newly designed *cyt b* PCR primers was determined using laboratory-reared *P. papatasi* females fed on human or chicken blood and maintained at 26°C. Sand flies were sacrificed at different times following the blood meal and processed for PCR. The signal strength of the amplified PCR products was optimal in flies sacrificed within the first 48 hours after feeding. However, *cyt b* DNA was clearly detectable up to and sometimes even exceeding 96 h after feeding (Fig. 2). Notably, the resulting PCR product (344 base pairs) was found to be unsuitable for discriminating between animals by RFLP analysis as required restriction sites were not found in the amplified segments.

Species-specific oligonucleotides

Eighteen oligonucleotide probes were designed to specifically hybridize with *cyt b* PCR products from different vertebrate species found in our study regions. Two additional oligonucleotides were designed to hybridize with any avian *cyt b* DNA in order to adequately separate bird blood, which is irrelevant to leishmaniasis and mammalian blood (Table 1).

Sensitivity of RLB hybridization

Cyt b PCR products were amplified from three different species (human, cow, and pigeon) using different concentrations of genomic target DNA. A sensitivity limit of 1 pg template per PCR reaction was achieved using DNA from the three vertebrate species (Fig. 3, top panel). The PCR products were also detectable by RLB hybridization (Fig. 3, bottom panel).

RLB analysis of the amplified cyt b PCR products from different vertebrates

Cyt b PCR products from different animal species were analyzed by RLB hybridization. About 1 ng genomic DNA obtained from known animal species was used in separate PCR amplification reactions (Fig. 4A). The PCR-amplified products were tested for their hybridization with the homologous oligonucleotides by RLB. Figure 4B represents RLB of the biotinylated *cyt b* PCR products to 20 oligonu-

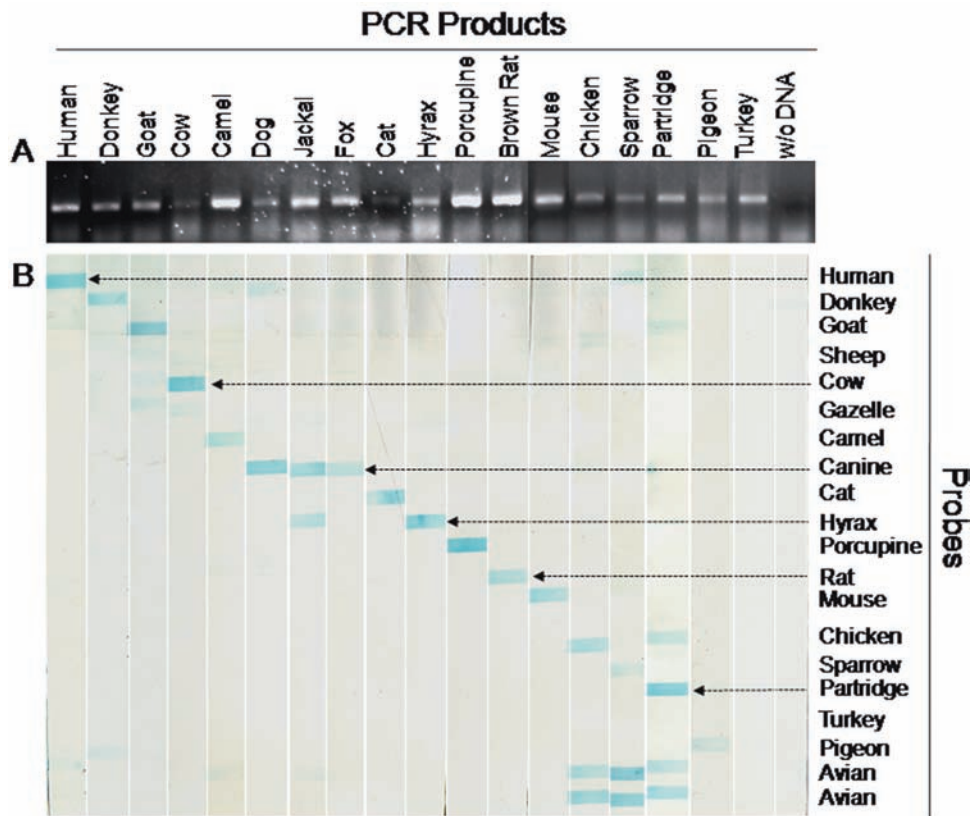


FIG. 4. Polymerase chain reaction (PCR)-reverse line blot (RLB) identification of cytochrome *b* DNA from different vertebrates—potential hosts of phlebotomine sand flies. (A) Cytochrome *b* PCR-amplified products from different vertebrates as indicated above the panel. (B) RLB hybridization analysis of the PCR products using the probes listed to the right of the panel.

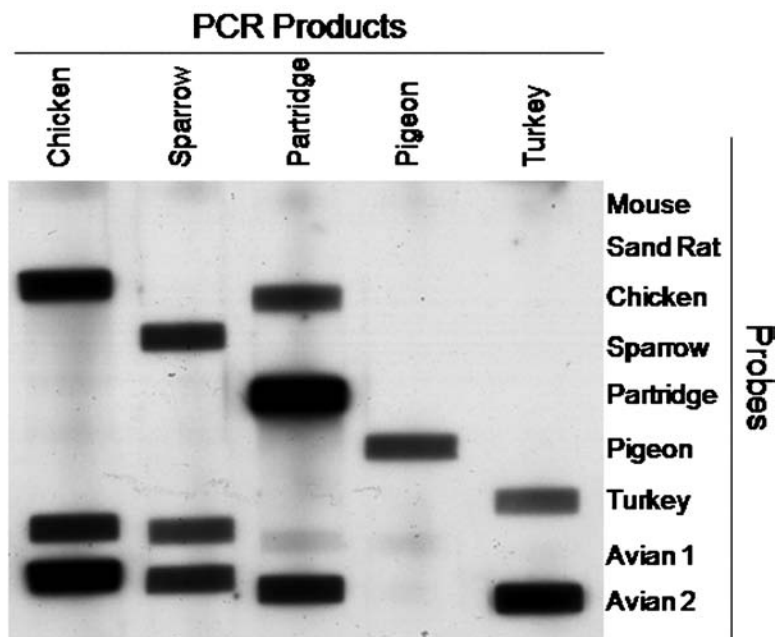


FIG. 5. Reverse line blot detection of avian polymerase chain reaction–amplified cytochrome *b* products using enhanced chemiluminescence. Lane 1, chicken; lane 2, bird; lane 3, partridge; lane 4, pigeon; lane 5, turkey; and lane 6, no DNA. M, size marker (bp).

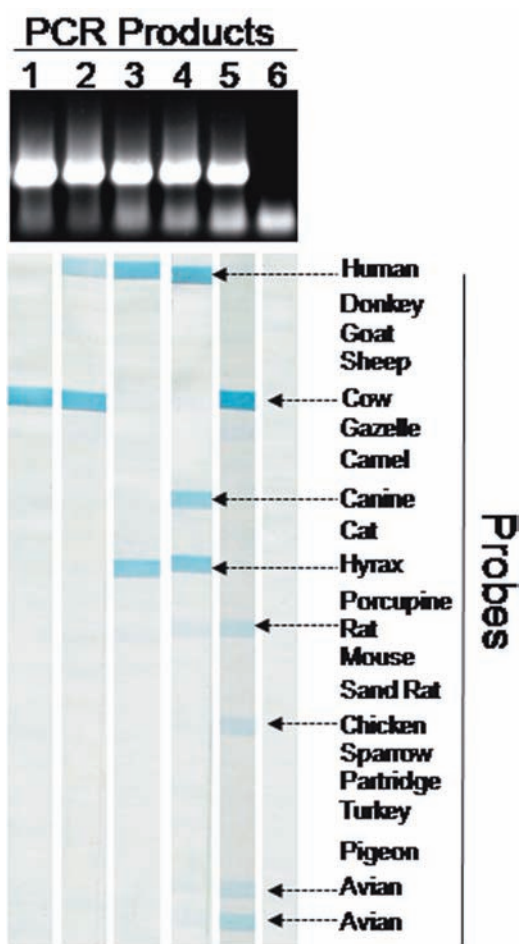


FIG. 6. Reverse line blot analysis of cytochrome *b* polymerase chain reaction products amplified from mixed samples of vertebrate DNA. Lane 1, cow only; lane 2, human and cow; lane 3, human and hyrax; lane 4, human, dog, and hyrax; lane 5, cow, rat, and chicken; lane 6, negative control (no DNA).

cleotides (Table 1) as detected using a colorimetric method. Each vertical strip contained 20 different species-specific probes in the order listed on the right side of Fig. 4B. Out of the 15 species-specific *cyt b* PCR products, the following hy-

bridized unambiguously only with their homologous oligonucleotide probes: human, donkey, cow, camel, cat, hyrax, porcupine, brown rat, and domestic mouse. Goat *cyt b* PCR products showed insignificant cross-hybridization with sheep and gazelle oligonucleotides. Owing to the close similarity in *cyt b* gene sequences of canine species (dog, jackal, and fox), designing species-specific oligonucleotides was unsuccessful. Therefore, only one oligonucleotide was used, which hybridized with *cyt b* PCR products from all canid species. Both dog and fox *cyt b* PCR products hybridized only with the canid probe, and jackal *cyt b* PCR product cross-hybridized with the hyrax-specific probe. The RLB was also efficient in discriminating between different avian species: chicken, sparrow, partridge, and pigeon. However, the signals were weak compared to those of mammals, and the turkey probe failed to hybridize in the experiment altogether (Fig. 4). To overcome the sensitivity problems, blots were processed for ECL using photographic film (Fig. 5). Under these conditions, chicken, sparrow, pigeon, and turkey *cyt b* PCR products hybridized exclusively to their oligonucleotide probes, and *cyt b* PCR product from partridge showed cross-hybridization with the chicken-specific probe. Most of the tested avian *cyt b* PCR products hybridized with at least one general avian oligonucleotide. However, the pigeon *cyt b* PCR product hybridized only with its specific probe (Fig. 5).

Mixed sample analyses

DNA extracted from different vertebrates was mixed in quantities likely to exist in arthropod blood meals and analyzed. PCR-RLB successfully identified species-specific *cyt b* templates in mixed DNA samples (Fig. 6).

Identification of blood meals of wild-caught sand flies

Blood meals ingested by wild-caught sand flies that had been kept frozen or kept at room temperature in 100% ethanol were identified using *cyt b* PCR-RLB. The source of blood was successfully identified in 68 of 89 sand flies tested (76%). Mixed blood meals were identified in 15 (17%) of those (Table 2). Some of these flies are depicted in Fig 7, where it can be clearly seen that one of the flies had fed upon both bovine and human blood. The PCR-RLB was able to discern the likely source of blood even in flies where the PCR product was barely visible on gels (e.g., Fig. 7, lanes 4-6).

TABLE 2. POLYMERASE CHAIN REACTION (PCR)-REVERSE LINE BLOT (RLB) BLOOD MEAL ANALYSIS OF FIELD-CAUGHT SAND FLIES*

Site	Sand fly species	Blood meal source (sample size)
Ma'ale Adumim (inside homes)	<i>Phlebotomus papatasi</i>	Human (34), avian (1), human and avian (1), human and mouse (1), negative (5); total 42
Bedouin farm (light traps)	<i>P. papatasi</i>	Cow (2), avian (1), cow and avian (6), cow and human (1), negative (9); total 19
Amnun (rock mounds, light traps)	<i>P. arabicus</i>	(3), hyrax (1), hyrax and mouse (3), negative (7); total 28

*Negative results mean failure to obtain a PCR product. All the PCR-positive samples were identified successfully using RLB.

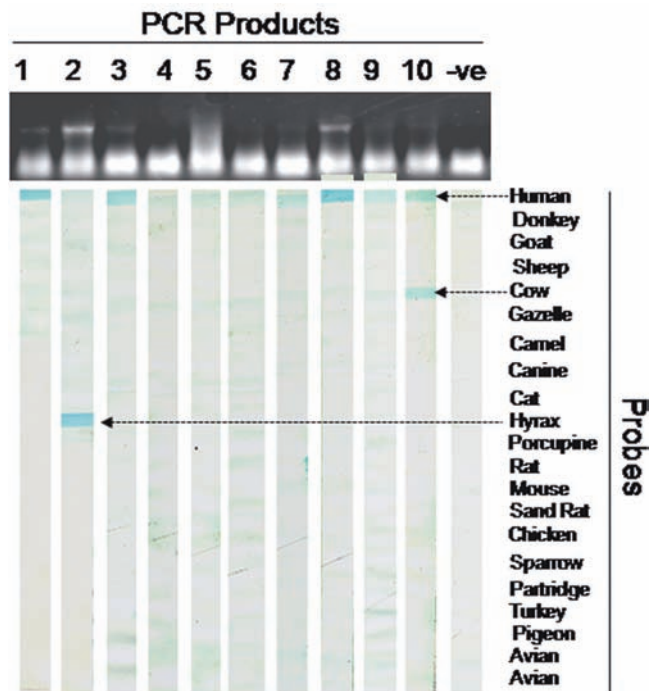


FIG. 7. Representative reverse line blot results of *cyt b* polymerase chain reaction products from blood-fed, field-caught sand flies. Samples number 1, 3, 7, and 8 demonstrate the presence of human DNA. Samples 4–6 fed human blood but blots were of poor quality. Sample 3 shows the presence of hyrax DNA. Sample 10 shows a mixture of human and cow blood meal. Sample 11 was unfed (= negative control). Samples 1–9 were collected near Amnun in the Galilee and identified as *Phlebotomus arabicus*, and samples 10 and 11 were collected near Ma'ale Adumim and identified as *P. papatasi*.

Discussion

To develop a rapid and sensitive technique for identifying the sources of blood meals of phlebotomine sand flies and other hematophagous arthropods, a relatively conserved part of the *cyt b* locus was amplified by PCR (Boakye et al. 1999, Mukabana et al. 2002, Meece et al. 2005). New primers were designed that increased sensitivity considerably, enabling the detection of very small quantities of DNA (Fig. 1). Sand fly blood meals were detectable up to 96 h postfeeding (Fig. 2), considerably longer than other published reports (Pichon et al. 2005, Haouas et al. 2007). In some cases, the RLB technique was useful even when the PCR amplified bands were hardly visible in the gels (Figs. 3 and 7). Importantly, such low quantities of DNA are not amenable to either RFLP analysis or sequencing, making RLB more sensitive than those two methods.

Results of the *cyt b* PCR-RLB indicated that availability of hosts played a decisive role in determining the species selected for feeding. For example, almost all of the *P. papatasi* females collected inside homes in Ma'ale Adumim, in which blood was identified (36/37), had fed on humans. On the other hand, *P. papatasi* females captured close to a small farm had fed on several farm animals (cows and chickens). Importantly, *P. arabicus* collected in rocky areas near homes in Amnun had fed on humans and wild animals including hyraxes (Table 2,

Fig. 7). This finding concurs with *P. arabicus* being the vector of *Leishmania tropica* in the Galilee region of northern Israel (Jacobson et al. 2003, Svobodova et al. 2006).

The major advantage of the PCR-RLB method, besides it being more sensitive than other methods, is its ability to differentiate between host species in mixed blood meals. During the PCR stage, there is nonspecific amplification of all vertebrate *cyt b* template DNA. During the RLB stage, any *cyt b* PCR product present in the mixture hybridizes separately with species-specific oligonucleotide probes attached to the membrane (Fig. 6). This quality makes PCR-RLB especially useful in studying blood meal composition in vectors of zoonotic diseases that tend to feed frequently (Fig. 6). In our preliminary study, PCR-RLB analyses indicated that the frequency with which sand flies in the wild feed on more than one host species is a lot higher than previously suspected. The tendency for feeding on more than one host may have important implications for disease transmission.

Recently, RLB was shown to be a very sensitive method for detecting *Leishmania* parasites and identifying their species (Naserddin and Jaffe, personal communication). Combining blood meal analysis with parasite and possibly sand fly identification in one Multiplex PCR-based RLB hybridization assay could provide valuable information on the vectorial role of different sand fly species and the reservoir status of different vertebrates (Kong and Gilbert 2006).

In conclusion, the *cyt b* PCR-RLB technique, which employs species-specific probes, proved suitable for identifying blood meals taken by sand flies (Figs. 3–5) and is a useful, timesaving approach for identifying blood meals of arthropods in situations where the number of potential hosts is limited (e.g., arid zones and peridomestic habitats). The method may also be applied in situations where the questions asked do not require the identification of many hosts and a basic distinction between two or three possibilities is sufficient (e.g., human versus nonhuman or mammalian versus avian). However, designing and applying species-specific probes for a large number of different hosts proved difficult to optimize, and several problems with cross-hybridization were not overcome satisfactorily in the current study (e.g., Fig. 4). Therefore, where there are a large number of potential hosts and each one requires a definite unambiguous identification, direct nucleotide sequencing would probably be a more appropriate choice.

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